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Article type : Regular Manuscript

Auxin-mediated statolith production for root gravitropism

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/nph.15932

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Tel: 86-29-85310288

Received: *28 March 2019*

Accepted: *11 May 2019*

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Key words: Auxin, Root gravitropism, statolith, gravity perception and response, *Arabidopsis*

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Summary

- Root gravitropism is one of the most important processes allowing plant adaptation to the land environment. Auxin plays a central role in mediating root gravitropism, but how auxin contributes to gravitational perception and the subsequent response is still unclear.
- Here, we showed that the local auxin maximum/gradient within the root apex, which is generated by the PIN directional auxin transporters, regulates the expression of three key starch granule synthesis genes, *SS4*, *PGM* and *ADG1*, which in turn influence the accumulation of starch granules that serve as a statolith perceiving gravity.
- Moreover, using the cvxIAA-*ccvTIR1* system, we also showed that TIR1-mediated auxin signaling is required for starch granule formation and gravitropic response within root tips. In addition, *axr3* mutants showed reduced auxin-mediated starch granule accumulation and disruption of gravitropism within the root apex.
- Our results indicate that auxin-mediated statolith production relies on the TIR1/AFB-AXR3-mediated auxin signaling pathway. In summary, we propose a dual role for auxin in gravitropism: the regulation of both gravity perception and response.

Introduction

Plants possess the ability to direct growth towards or away from external stimuli such as light, water, temperature and gravity. These directional growth responses are termed tropisms. More than a century ago, Charles Darwin studied plant tropism in detail, showing that etiolated grass seedlings grow towards the light when they are illuminated from one side (Darwin, 1880). Among the tropisms, gravitropism has been one of the most extensively studied because it is critical for the ability of roots to perceive gravity, anchor in the soil and

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navigate heterogeneous soil environments in order to secure water and nutrients (Bailey, 2002).

Root gravitropism can be divided into three temporally distinct phases: gravity perception, transmission of the gravitropic signal and ultimately the growth response itself (Swarup & Bennett, 2009). The root tip is the primary site of gravity sensing in higher plants, and surgical removal of the root cap results in agravitropic roots (Blancaflor & Masson, 2003; Morita & Tasaka, 2004). The root cap is comprised of central columella cells and surrounding root cap cells (Dolan *et al.*, 1993), and its patterning and formation relies on the auxin signaling (Sabatini *et al.*, 1999). In *Arabidopsis* (*Arabidopsis thaliana*), the columella cells consist of four cell layers termed S1, S2, S3 and S4. Sophisticated laser ablation experiments revealed that the S1 and S2 layers, which are adjacent to the distal stem cells, appear to be the most important layers for gravity perception (Blancaflor *et al.*, 1998). The perceptional capability of the columella cells might be due to the starch-filled plastids (amyloplasts) that accumulate within these S1 and S2 cells. The synthesis of starch biosynthesis begins with the conversion of glucose-6-phosphate into glucose-1-phosphate catalyzed by plastidial phosphoglucomutase (PGM). Adenosine diphosphate glucose pyrophosphorylase (ADG) subsequently converts glucose-1-phosphate and ATP into ADP-glucose, which was further converted into starch by granule bound starch synthase1 (GBSS1) and a series of starch synthases (SS1, SS2, SS3 and SS4) (Bahaji *et al.*, 2014).

The starch-statolith hypothesis postulates that the sedimentation of dense and starch-filled amyloplasts triggers gravity signal transduction, which results in a graviresponse in the elongation zone and gravitropic bending (Leitz *et al.*, 2009; Band *et al.*, 2012a; Baldwin *et al.*, 2013; Blancaflor, 2013; Sato *et al.*, 2015). Pharmacological-, genetic-, and cell biology-based approaches have shown that the signal mediating gravitropic response is auxin. After gravity sensing, the auxin efflux facilitators PIN3 and PIN7 relocalize to the lower side of the columella cells and mediate auxin redistribution (Friml *et al.*, 2002b; Kleine-Vehn *et al.*, 2010). Once auxin asymmetry is established by PIN3 and PIN7, auxin is transported by

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AUX1/PIN2 in a shootward direction through the lateral root cap to the epidermal cells in the elongation zone (Blilou *et al*., 2005). Visualization of auxin response reporter DR5 (a mutated 5' end of the D1-4 auxin response element) and DII (an Aux/IAA-based reporter, domain II) activity revealed that auxin differentially accumulates between the upper and lower parts of the root tips after gravistimulation (Muller *et al*., 1998; Galweiler *et al*., 1998; Brunoud *et al*., 2012; Swarup and Bennett, 2009). The epidermal cells are thought to be the main mediators of the gravitropic response triggered by this unbalanced distribution of auxin because blocking the auxin response specifically in these cells results in the loss of gravitropic bending (Wong *et al*., 1996).

Mutations in several auxin signaling components results in agravitropic roots, such as those of the *axr3* and *tir1/afb* mutants in which the auxin signaling pathway is disrupted (Nakamura *et al*., 2006; Baster *et al*., 2013). The *TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX* (*TIR1/AFB*) genes encode F-box proteins, which are able to interact with the cullin CUL1 and the S-phase Kinase-Associated Protein (SKP) 1-like proteins to form the SKP1-Cullin-F-box (SCF) ubiquitin protein ligase (E3). SCF^{TIR1} interacts with AUX/IAA proteins, such as AXR2/IAA7 and AXR3/IAA17 (Gray *et al*., 2001). SCF^{TIR1} contains leu-rich repeats and an auxin binding pocket (Tan *et al*., 2007). The binding of auxin in this pocket is stabilized by the docking of the Aux/IAA proteins. It has been reported that SCF(TIR1/AFB)-auxin signaling mediates root gravitropism by regulating PIN vacuolar trafficking and auxin fluxes (Baster *et al*., 2013). Gene expression studies have shown that after only 15 min of gravistimulation, several auxin-inducible genes, including *IAA1*, *IAA2* and *ARF19*, are significantly upregulated (Band *et al*., 2012b; Brunoud *et al*., 2012). Moreover, there is evidence for a secondary auxin-independent gravity response mechanism in *Arabidopsis*; this mechanism may include other signaling molecules, such as protons, Ca^{2+}, and inositol 1,4,5-triphosphate (Lee *et al*., 1983; Monshausen *et al*., 2011).

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Although it has long been known that auxin is critical for mediating the gravitropic response, the final step of gravitropism, it is still not clear whether auxin also takes part in gravity perception, the initial step of gravitropism. In this study, we show that in addition to its important role in the response to gravity signals triggered by starch granules, auxin also regulates the synthesis of starch granules within the root apex, suggesting that it also has an important role in the perception of the gravity signal. Our work highlights the dual role of auxin in mediating the gravitropic response.

Materials and Methods

Plant lines and growth conditions.

The *Arabidopsis* single mutant *pin2* (Col-0) and the triple mutant *pin3-5/pin4-3/pin7-1* (Col-0/Ler) were previously described (Leitner *et al*., 2012; Benkova *et al*., 2003). The *yuc1/2/4* (Col-0), *yucca1D* (Col-0), and *tir1-1/afb2-1/afb3-1* (Ws) mutants and the *HS::axr3-1* (Col-0) and *ccvTIR1* lines (Ws) were also previously described (Cheng *et al*., 2006; Knox *et al*., 2003; Dharmasiri *et al*., 2005a). The *DR5::GFP* reporter gene (Swarup and Bennett, 2009) was introduced into these mutant lines by crossing. The starch granule synthesis mutant lines were obtained from the Nottingham *Arabidopsis* Stock Centre: *ss1* (Col-0), N624597; *ss2* (Col-0), N653660; *ss3* (Col-0), N573715; *ss4* (Col-0), N2107324; *Gbss1* (Col-0), N554502; *adg1* (Col-0), N3094 and *pgm* (Col-0), N210. The *Arabidopsis* mutant line *axr3-1* (Col-0; CS57505), which has a single amino acid substitution (Nakamura *et al*., 2006), was obtained from the SALK collection (http://signal.salk.edu) at the *Arabidopsis* Biological Resource Center, USA. Seeds were surface sterilized with 0.1% $HgCl_2$ and sown on Murashige and Skoog (MS) medium. After 2 weeks, seedlings were transferred to soil, and grown in an Intellus control system (Percival) with a 16/8-h light/dark cycle at 22°C in 70% humidity (Zhang *et al*., 2015). For microscopic analyses of gravitropism, seedlings grown or transferred in Petri dishes containing half strength MS (from Sigma, M5524) medium with

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0.8% Agar (Sigma, A7921) and 1% sucrose were gravi-stimulated by rotating the stage 135° of the plates for the specified amount of time before imaging. The degree of bending angle (mean ± SD) was measured by the Fiji software and calculated from the primary roots of 10 independent plants for each genotype.

qRT-PCR analysis

RNA was extracted from root apices harvested from 7-day-old *Arabidopsis* seedlings as previously described (Zhang *et al.*, 2015), and cDNA was reverse-transcribed from 5 μg of total RNA according to the manufacturer's instructions (TaKaRa Biotech, Dalian, China). For each gene, qRT-PCR assays were performed for three biological and three technical replicates using the following parameters: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 54°C for 20 s. A melting curve was generated from 65°C to 95°C. Statistical significance was evaluated by Student's t-test. The *AtUBQ5* gene was used as the internal control for all qRT-PCR analyses (Table S1).

Starch staining

To observe the starch granules in the root tips, twenty *Arabidopsis* roots (7-day old) were dipped in Lugol's staining solution (Sigma-Aldrich) for 5 min, washed with distilled water and then observed under a differential interference contrast microscope (Leica DMRE).

For the mPS-PI staining (Truernit *et al.*, 2008), whole seedlings were fixed in 50% methanol/10% acetic acid at 4°C for up to 24 h. The tissue was rinsed briefly with ddH_2O and incubated in 1% periodic acid at room temperature for 40 min. The tissue was then rinsed twice with ddH_2O and incubated in Schiff's reagent containing propidium iodide (100 mM sodium metabisulphite, 0.15 N HCl, and 100 mg/mL propidium iodide) for 2 h until the plants were visibly stained. More than three samples were transferred onto microscope slides and

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covered with chloral hydrate solution (4 g chloral hydrate, 1 mL glycerol, and 2 mL water). The slides were kept overnight at room temperature, after which excess chloral hydrate was removed. The seedlings were mounted in Hoyer's solution (30 g gum arabic, 200 g chloral hydrate, 20 g glycerol, and 50 mL water). The slides were left undisturbed for at least 3 d before observation (excitation 488 nm, emission 520 to 720 nm).

Confocal microscopy. For confocal microscopic analyses, 7-day-old seedlings grown in half-strength MS medium were stained with 10 μg/mL propidium iodide for 5 min (Sarkar *et al.*, 2007), washed briefly in ddH_2O and visualized at 600-640 nm for propidium iodide and 500-560 nm for GFP on an LSM 710 NLO confocal microscope with Duoscan. The *DR5::*GFP signal intensity of the columella region containing the GFP signal was quantified by the measurement of the mean gray value with the Fiji software (https://imagej.net/Fiji).

RESULTS

Auxin affects starch granule accumulation by regulating starch synthase genes within the root apex

First, we investigated whether the local auxin maximum in the root tip, established by PIN protein-mediated directional auxin transport (Friml *et al.*, 2002b), affects starch granule formation. We exogenously applied the auxin analogue 1-Naphthaleneacetic acid (NAA) to enhance the concentration of auxin in the root tips and used the synthetic auxin-responsive promoter reporter *DR5::GFP* to evaluate auxin accumulation and Lugol's staining to evaluate starch granule accumulation. We found that roots tips treated with 1 μM NAA had much stronger *DR5::GFP* signals and much more starch granule accumulation when compared with root tips treated with DMSO (Fig. 1a, b; Fig. S1a, b). The modified propidium iodide staining (mPS-PI) (Horrer *et al.* 2016) further indicated that the most obvious feature of the

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amyloplast is their significantly increased size within the columella cells (Fig. 1a, b; Fig. S2a). N-1-naphthylphthalamic acid (NPA), which also increases the auxin level within the root apex (Friml *et al*., 2002a; Yamada *et al*., 2009), similarly enhanced starch granule formation (Fig. 1c; Fig. S1c, S2a), further confirming that auxin within the root apex facilitates the accumulation of the starch granules. However, when plants were treated with the auxin synthesis inhibitor L-Kynurenine (L-Kyn) (He *et al*., 2011), *DR5::GFP* signal and amyloplast size were dramatically decreased, indicating the starch granule formation was severely inhibited (Fig. 1d; Fig. S1d and S2a). To confirm that the reduced size of amyloplasts within the root apex was mainly caused by the decrease in the auxin maximum and not by other side-effects of L-Kyn, we treated the seedlings with both NAA and L-Kyn. We found that the dramatically reduced *DR5::GFP* intensity and starch granule accumulation observed after L-Kyn treatment could be restored by extra application of NAA (Fig. 1e; Fig. S1e and S2a). Moreover, the *DR5::GFP* intensity and starch granule accumulation in the roots treated with the both NAA and L-Kyn were similar to those in roots treated only with NAA (Fig. 1b, e and f).

The homeostatic starch granule accumulation depends on the balance of starch synthesis and degradation, which is regulated by distinct sets of genes (Sonnewald and Kossmann, 2013; Silver *et al*., 2014) (Fig. S3). To determine which pathway and genes are mainly activated by auxin signaling to regulate starch granule accumulation, we used quantitative real-time PCR (qRT-PCR) to determine the expression levels of starch granule-related genes. Application of NAA or NPA significantly upregulated genes involved in the starch granule synthesis pathway, especially the key starch granule synthesis genes *PGM*, *ADG1* and *SS4* (Fig. 1g; Fig. S4). In contrast, there was little effect on the expression of starch granule degradation genes, except for *BAM3*, whose expression was small induced by NAA treatment (Fig. S4). Furthermore, exogenously applied L-Kyn notably downregulated the expression of *PGM*, *ADG1* and *SS4*, but had no significant or slightly effect on the transcriptional levels of other starch granule synthesis genes or genes involved in starch

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degradation pathways (Fig. 1g; Fig. S4). The reduced auxin resulted from L-Kyn treatment would inhibit the starch synthesis (Fig.1), while the degradation rate kept the same (Fig. S4), thereby resulting in the reduced starch granule accumulation in the root tips.

We next used mutants of the *YUC* genes, which are auxin synthesis genes controlling auxin production, to confirm the function of auxin in starch granule synthesis and accumulation. Compared with the wild type, the triple mutant *yuc1/2/4* showed less starch granule accumulation with reduced size of these amyloplasts as well as down-regulated expression of *PGM*, *ADG1* and *SS*4 (Fig. 1h,i,k; Fig.S2b), whereas the gain-of-function mutant *yucca1D*, which has enhanced auxin biosynthesis, showed much higher transcriptional levels of the three key starch granule synthesis genes and much more starch granule accumulation within the root apex (Fig. 1j,k; Fig.S2b).

These results from both *in vivo* and *in vitro* experiments strongly suggest that auxin regulates the starch granule synthesis pathway and the starch granule biosynthesis genes *PGM*, *ADG1* and *SS4,* which in turn regulate starch granule accumulation in the root apex. Furthermore, *cis*-element analysis showed that *PGM* and *ADG1* promoters contained the auxin responsive element (ARE, Fig. S5), indicating that the auxin response factor might bind at these AREs to regulate their expression directly, whereas the *SS4* promoter does not contain the auxin responsive element within 2 Kb, suggesting an indirect effect of auxin signaling on the regulation of its expression.

Endogenous alteration of the auxin maximum within the root apex also affects starch granule accumulation

To further confirm that starch granule accumulation is regulated by auxin in the root tip, we endogenously modified auxin maxima by knocking out the *PIN* auxin transporter genes, which mediate directional auxin transport and are important for establishing root auxin maxima as well as for root gravitropism (Fig. 2a; Fig. S6) (Adamowski, *et al.*, 2015). *PIN2* is

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mainly responsible for the outward transport of auxin that accumulates in the root apex (Wišniewska *et al.*, 2006). Compared with wild type, the loss-of-function mutant *pin2* had an enhanced auxin maximum in the root tip as indicated by *DR5::GFP* signal intensity (Fig. 2b, c; Fig. S7a, b). In addition, many more starch granules accumulated with substantially increased size within the root apex of the *pin2* mutant than that in wild type (Fig. 2c and Fig. S8), and the starch granule synthesis genes *PGM*, *ADG1* and *SS4* were also upregulated (Fig. 2h). *PIN1/3/4/7* are responsible for the inward transport of auxin to the root apex through the stele (Fig. 2a). Compared with wild type, the *pin3/4/7* triple mutant had slightly reduced *DR5::GFP* signal and starch granule accumulation indicated by their much smaller sizes in the root apex (Fig. 2d; Fig. S7c and S8). However, notably in the *pin3/4/7* triple mutant, the expression zone of the *DR5::GFP* is enlarged in the root tip and expanded to the adjacent statocytes compared with the wild type (Fig. 2b). Decreased gene expression in an expanded domain of the root cap may explain why the expression levels of the starch synthesis genes *PGM*, *ADG1* and *SS4* were not significantly different between *pin3/4/7* and wild type (Fig. 2h).

We next treated the *pin2* mutant with L-Kyn. The enhanced auxin maximum in this mutant was dramatically decreased as indicated by *DR5::GFP* signal intensity, and there was lower expression of the three key starch granule biosynthesis genes (*SS4*, *PGM* and *ADG1*) and smaller starch granule within the root apex (Fig. 2e,h; Fig. S7d and S8). When we applied NAA to enhance the auxin maximum in the *pin3/4/7* mutant, the *DR5::GFP* signal intensity was significantly increased, the size of amyloplasts within the root apex was increased and the expression of *SS4*, *PGM* and *ADG* was upregulated (Fig. 2f,h; Fig. S7e and S8). These results provide further evidence that the auxin maximum, generated by PIN-mediated transport, regulates starch granule formation within the root apex by regulating the expression of starch synthesis genes.

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Alteration of starch granule accumulation influences root gravitropism

After treatment with NAA, NPA or the auxin biosynthesis inhibitor L-Kyn for 48 h, we washed the treated seedlings with ddH_2O to remove residual chemicals, transferred them to new medium, grew them without any treatment for two days, and then analyzed root gravitropism. Seedlings pretreated with NAA or NPA showed dramatically more starch granule accumulation within the root apex compared with seedlings pretreated with DMSO (Fig. 3a-c). After gravistimulation, the *Arabidopsis* roots pretreated with NAA or NPA showed much faster gravitropism compared with those pretreated with DMSO (Fig. 3f; Fig. S9), indicating that auxin-promoted starch granule accumulation within the root apex influences subsequent root gravitropism. However, the seedlings pretreated with L-Kyn showed reduced starch granule accumulation within the root apex and slower gravitropism relative to the DMSO-treated control, even though the signals intensities of *DR5::GFP* between different treatments were comparable (Fig. 3d and 3f; Fig. S10). Similar to roots pretreated with NAA, roots pretreated with both NAA and L-Kyn showed enhanced starch granule accumulation and accelerated root gravitropism (Fig. 3e and 3f), which further supported our conclusion that the alteration of starch granule accumulation by auxin changes the speed of root gravitropism.

Starch granule synthesis genes that are significantly regulated by auxin are critical for starch granule accumulation in the root apex

We have shown that the auxin maximum generated by the PIN proteins regulates starch granule accumulation and the expression of the starch granule synthesis genes *PGM*, *ADG1* and *SS4*. To verify that these genes play an important role in starch granule formation within the root apex, we observed the phenotypes of plants harboring mutations in *PGM*, *ADG1* and *SS4* as well as in other starch synthesis genes whose expression was not affected by auxin, including *STARCH SYNTHASE 1* (*SS1*), *SS2*, *SS3*, and *GBSS1* (Fig. S4). The accumulation

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of starch granules in the root apices of the *ss1*, *ss2*, ss3, and *Gbss1* mutants determined by Lugol's and mPS-PI staining was comparable to that in the wild type (Fig. 4a-e; Fig. S11). However, in the *ss4* mutant, amyloplast size was significantly reduced (Fig. 4f; Fig. S11), and in the *adg1* and *pgm* mutants, the root apices were devoid of starch granules (Fig. 4g, h). These results suggest that starch granule synthesis genes that are significantly regulated by auxin do play vital roles in starch granule accumulation in the root apex. Consistent with the loss of starch granules, which serve as statoliths perceiving gravity, the *ss4*, *pgm* and *adg1* mutants also showed severe defects in gravitropism that were not observed in wild type and the other starch granule synthase mutants *ss1*, *ss2*, ss3 and *Gbss1* (Fig. 4i; Fig. S12).

To further confirm that the starch granule synthase genes *SS4*, *PGM*, and *AGD1* are the key genes regulated by auxin and that they function downstream of auxin signaling, we treated the *ss4*, *pgm* and *adg1* mutants with NAA. After NAA treatment, the root apices of the *adg1* and *pgm* mutants were still devoid of starch granules. However, the NAA-treated *ss4* roots still showed much more starch granules than the *ss4* roots without the treatment of the NAA, even if their starch granule accumulation was lower than that in the wild type (Fig. S13).

Auxin regulates starch granule accumulation within the root apex through the TIR/AFB-mediated pathway.

We next wanted to know how auxin functions in starch granule accumulation. The TIR1/AFB F-box proteins are auxin receptors that bind auxin and mediate signaling downstream of auxin (Dharmasiri *et al.*, 2005a). Compared with the wild type, the *tir1/afb2/afb3* triple mutant showed less starch granule accumulation with smaller size and reduced expression of the key starch granule synthesis genes *SS4*, *PGM* and *ADG1* (Fig. 5a-c; Fig. S14a). Moreover, after NAA treatment, the *tir1/afb2/afb3* mutant showed reduced amyloplast size than NAA-treated wild type, and the expression levels of the *SS*4, *PGM* and *ADG1* genes in *tir1/afb2/afb3* were also lower than those in the wild type (Fig. 5a-c; Fig. S14a). These results

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indicate that the TIR1/AFB-mediated auxin signaling pathway plays an important role in starch granule accumulation within the root apex.

To verify that auxin activation of the TIR-pathway regulates starch granule accumulation, we used the newly developed cvxIAA-*ccvTIR1* (convex IAA- concave TIR1) system as previously reported (Uchida *et al.*, 2018). The chemical compound cvxIAA specifically binds an engineered version of TIR1, *ccvTIR1*, to activate the TIR1/AFB-mediated auxin signaling pathway. Compared with the wild type, transgenic plants carrying *ccvTIR1* showed significantly increased amyloplast size and the expression of *SS4*, *PGM* and *ADG1* when treated with cvxIAA (Fig. 5d-f; Fig. S14b). To test whether TIR1/AFB-mediated starch granule formation within the root apex is important for regulating gravitropism, we pretreated the transgenic line *ccvTIR1* with cvxIAA or DMSO and evaluated gravistimulation. Compared with the DMSO control, the *ccvTIR1* line pretreated with cvxIAA showed substantially more starch granule accumulation and much faster gravitropism (Fig. S15). These results indicate that the regulation of starch granule accumulation by auxin within the root apex and root gravitropism depends on the TIR1/AFB pathway.

Moreover, to further confirm the starch granule accumulation rely on the TIR1/AFB-mediated auxin signaling in the root statocytes, we used the promoter of *cellulose 5* (*AtCEL5*), an endo-β-1,4-D-glucanase gene that exclusively expressed in the root cap (Campillo, *et al.* 2004), to specifically drive the expression of the *ccvTIR1* in the root apex. After the treatment of the transgenic line *pCEL5::ccvTIR1* with cvxIAA, it is clearly shown that the three key starch granule synthesis genes (*PGM*, *ADG1* and *SS4*) are significantly upregulated, and thus the starch granules are substantially accumulated with increased size (Fig. S16 and S17).

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AXR3/IAA17 regulates starch granule accumulation by mediating auxin maximum-triggered signaling

It has been reported that TIR1 interacts with AXR3/IAA17 to mediate auxin signaling (Gray *et al.*, 2001). AXR3/IAA17 is a short-lived transcriptional factor that functions as a repressor of early auxin response genes (Tiwari *et al.*, 2004). In the gain-of-function mutant *axr3-1*, which encodes a variant of AXR3 with increased stability (Rouse *et al.*, 1998), auxin signaling is severely blocked, which is evident from the defective expression pattern of *DR5::GFP* in *axr3-1* (Fig. 6a,b). Furthermore, starch granule accumulation and gravitropism are abolished in *axr3-1* mutant (Fig. 6c,d ; Fig. S18), and the expression of *PGM*, *ADG1* and *SS4* is significantly downregulated in *axr3-1* mutant compared with wild type (Fig. 6e). To further confirm the function of IAA17/AXR3 in auxin-mediated starch granule accumulation, we analyzed starch granule accumulation and starch synthesis gene expression in the heat inducible line *HS::axr3-1*. The root cap cells of wild type contained the amyloplasts of similar sizes whether exposed to 25°C or 37°C, and expression of *SS4*, *PGM* and *ADG1* genes was also similar between these treatments (Fig. 6f, h; Fig. S19). However, compared with the roots of untreated *HS::axr3-1,* the roots of *HS::axr3-1* exposed to 37°C heat shock treatment, showed significantly decreased starch granule formation and much lower expression of *SS4*, *PGM*, and *ADG1* (Fig. 6g,i; Fig. S19).

To investigate the structure of *axr3-1* roots, we introduced a columella-specific enhancer trap, Q1630 (Sabatini *et al.*, 1999), into *axr3-1* mutant. The result showed that the root apex of *axr3-1* mutant also seemed to contain the columella-like cells (Fig. 6j). To test the hypothesis that the reduced *DR5::GFP* signal in *axr3-1* is caused by disruption of auxin signaling within the root apex and not by abnormal *PIN* expression, we first performed qRT-PCR analysis of the *PIN* genes in the *axr3-1* mutant. We found that the *PIN* transcriptional levels in *axr3-1* were almost identical to those in wild type (Fig. 6k). Furthermore, the accumulation of the PIN1-GFP and PIN2-GFP proteins in *axr3-1* and wild type was also almost identical (Fig. 6l,m). These results strongly suggest that auxin maxima

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might still be formed in *axr3-1* and that the reduction in *DR5::GFP* signal in the root tip of this mutant may be due to the disruption of auxin signaling.

We next tested the auxin response in the S1-S4 columella cells of *axr3-1* by exogenously applying the auxin analogue NAA. In contrast to the notably enhanced *DR5::GFP* signal intensity in wild-type plants after NAA treatment, the decreased *DR5::GFP* signal in *axr3-1* mutant root tips was not affected by NAA treatment (Fig. 6n,o), indicating that the auxin response in the *axr3-1* root apex is defective. Consistent with this, no starch granule formation was observed in the root apex of *axr3-1* mutant after NAA treatment (Fig. 6p; Fig. S20). In order to reveal the important role of the AXR3-mediated auxin signaling in the regulation of the starch granules accumulation in root apex, we used the root-cap-specific promoters *pCEL5* to drive the expression of *AXR3-1* to exclusively disrupt this auxin signaling pathway within the root apex. In contrast to wild type plants, the transgenic plant *pCEL5*::*AXR3-1* showed no starch granule, reduced gravitropism and much lower expression of *SS4*, *PGM*, and *ADG1* in the root caps (Fig. S21). These results suggest that the regulation of starch granule synthesis and accumulation by the auxin maximum in the root apex is mediated by the AXR3 pathway.

Discussion

Gravitropism can be divided into three spatially and temporally distinct phases: gravity perception, transmission of the gravitropic signal, and ultimately the growth response itself. It has long been known that starch granules, which accumulate in root tips and act as statoliths, endow plants with the ability to transform a perceived gravity signal into an auxin signal that directs plant root growth toward the gravity vector (Sato *et al*., 2015). Our results show that in addition to its function in the response to the perceived gravity signal, auxin is also essential for regulating starch granule accumulation in the root apex and gravity perception (Fig. 7). Thus, we propose that auxin has a dual role in root gravitropism. There are six AFBs and

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more than twenty Aux/IAAs in *Arabidopsis*. TIR1/AFB-Aux/IAA pairs are always considered to be co-receptors for auxin. Different TIR1/AFB-Aux/IAA pairs have different affinities for each other. Our results suggest that auxin regulates starch granule formation via the TIR1-AXR3 pair, which regulates the key starch granule synthesis genes *PGM*, *ADG1* and *SS4*.

There are four homologous *STARCH SYNTHASE* (*SS*) genes, *SS1*, *SS2*, *SS3* and *SS4* (Seung *et al*., 2016). Of these genes, *SS4* seems to play a predominant role in starch granule formation within the root apex and in root gravitropism. Recent work has also shown that *SS4* gene is important for regulating starch granule number in leaves (Malinova *et al*., 2017). Further work revealed that among SS isoforms, only SS4 contains a unique N-terminal region with several long coiled-coil motifs that have been implicated in protein-protein interactions. This N-terminal region targets SS4 to the correct subcellular location and is necessary for the establishment of the correct granule morphology (Lu *et al*., 2018). Here, we found that no single *SS* mutant showed a complete lack of starch granules within the root apex, such as that observed in the *pgm* and *adg1* mutants, and we cannot exclude the possibility of functional redundancy between the *SS* genes in the regulation of starch granule accumulation within the root apex. This hypothesis can be tested in future analysis of multi-mutant lines of *SS* genes.

Our finding that AXR3-mediated auxin signaling regulates starch granule accumulation in gravity-sensing columella cells suggests that AXR3 indirectly regulates gravitropic perception. A previous study also found that AXR3 plays a critical role in gravity signal transduction and response: expression of a dominant mutant form of this auxin response repressor, axr3, in expanding epidermal cells located in the gravity-responsive elongation zone abolished root gravitropism (Swarup *et al*., 2005). These results strongly suggest that the dual role of auxin in gravitropism relies on AXR3 signaling in the columella cells at the root tip and in epidermal cells in the elongation zone.

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Our work highlights the dual role of auxin in mediating plant gravitropism and suggests that the auxin gradient/signaling within the root apex is able to regulate the starch granule accumulation and thus control the root gravitropism in terms of the results with the study of the speed of root curvature response. However, it remains to be determined if the root gravisensitivity is regulated by the starch granules accumulation within the root apex. Additionally, it is unknown if the same working model is also employed by the plant shoot to elaborately regulate its gravitropism, and unraveling it will be helpful to reveal the evolutionary conservation between the shoot and root gravitropism. Notably, recently, a centrifugal device combined with growth kinematic imaging was developed and revealed that shoot gravitropism is stimulated by sensing inclination not gravitational force as previously believed (Chauvet *et al.*, 2016). This newly developed method also would be very helpful to reveal the root gravisensitivity by amyloplasts settling in the root statocytes as well as its working mechanism in the future work. Gravitropism has an important impact on agriculture because it allows plants to compete for the limited resources available in their immediate environment. A better understanding of plant gravitropism will be important for future agricultural and horticultural applications.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 31600223), Hong Kong Scholars Program (XJ2017017), the Fundamental Research Funds for the Central Universities (Grant No. GK201803041, GK201702016 and GK201901004), the State Key Laboratory of Cotton Biology Open Fund (Grant No. CB2018A03) and the IST Fellow grant.

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AUTHOR CONTRIBUTIONS

G.X. designed the research. Y.Z. and P.H. performed the research. X.M., Z.Y., J.Y., C.P. and G.W. analyzed the data. G.X., Y.Z. and J.F. wrote the paper. Y.Z., P.H. and X.M. contributed equally to this work.

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Figure legends

Fig. 1 Altered auxin maximum/production influences starch granule accumulation within the root apex. (a-e) Analysis of *DR5::GFP* expression (upper panel) and starch granule accumulation (middle/lower panels) in the root apices of 7-day-old wild-type *Arabidopsis* seedlings. The level of *DR5::GFP* expression is reflected by signal intensity. The GFP channel images in the upper panel are shown in pseudocolor, and the intensity scale is shown at the right of each image (H, high; L, low). Starch granule accumulation was evaluated by staining with Lugol's solution (middle panel) and modified propidium iodide staining (mPS-PI) staining (lower panel). (a) Treatment with DMSO as the control. (b) Treatment with 1 μM auxin analogue NAA for 48 h. (c) Treatment with 20 μM auxin transport inhibitor NPA for 48 h. (d) Treatment with 25 μM auxin inhibitor L-Kyn for 48 h. (e) Treatment with both 25 μM L-Kyn and 1 μM NAA for 48 h. (f) Quantification of the *DR5::GFP* signal intensity in wild-type *Arabidopsis* seedlings treated with DMSO, 1 μM NAA, 20 μM NPA, 25 μM L-Kyn, or 25 μM L-Kyn/1 μM NAA. (g) The transcript levels of three key starch granule synthesis genes, *PGM*, *ADG1* and *SS4*, in wild-type root tips after treatment with NAA, NPA, L-Kyn or both L-Kyn/NAA. (h-j) Lugol's staining (upper panel) and mPS-PI staining (lower panel) of the root apex of 7-day-old *Arabidopsis* seedlings. (h) wild type (WT). (i) the loss-of-function *yuc1/2/4* triple mutant. (j) the gain-of-function *yucca1D* mutant. (k) The transcript levels of *PGM*, *ADG1* and *SS4* in root tips of wild type and the *yuc1/2/4* and *yucca1D* mutants. Error bars represent ±SD from three biological replicates. Asterisks denote the statistical significance of comparisons with DMSO-treated wild type: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Bars, 20 μm.

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Fig. 2 Starch granule accumulation in *pin* mutants with altered auxin maxima. (a) Schematic diagram of the generation of an auxin maximum by the PIN auxin transporters. (b-f) Auxin maxima indicated by *DR5::GFP* expression (upper panel) and starch granule accumulation indicated by Lugol's and mPS-PI staining (middle and lower panels). b, Wild type (WT). (c) Enhanced auxin maximum and starch granule accumulation in the *pin2* mutant. (d-e) Reduction in the auxin maximum (upper panel) and starch granule accumulation with smaller sizes (middle and lower panels) in the *pin3/4/7* triple mutant (d) and the *pin2* mutant (e) treated with 25 μM L-Kyn for 48 h. (f) Enhanced auxin maximum and starch granule accumulation in the *pin3/4/7* triple mutant after treatment with1 μM NAA for 48 h. In (b) to (f), the GFP channel images (upper panel) are shown in pseudocolor, and the intensity scale is shown at the right of each image (H, high; L, low). (g) Quantification of the *DR5::GFP* signal intensities in (b) to (f). (h) qRT-PCR analysis of three key starch granule synthesis genes, *PGM*, *ADG1* and *SS4*, in the root tips of wild type, *pin2* and *pin3/4/7;* the *pin2* mutant treated with 25 μM L-Kyn; and the *pin3/4/7* triple mutant treated with1 μM NAA. Error bars represent the ±SD from three biological replicates. Asterisks denote the statistical significance of comparisons with wild type: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. Bars, 20 μm.

Fig. 3 Altered starch granule accumulation after pretreatment with an auxin analogue or an auxin synthesis inhibitor affects root gravitropism. (a-e) Analysis of starch granule accumulation in *Arabidopsis* roots with Lugol's staining after pretreatment with DMSO (a), 1 μM NAA (b), 20 μM NPA (c), 25 μM L-kyn (d) and both 1 μM NAA and 25 μM L-Kyn (e). (f) Analysis of root gravitropism after the treatment shown in Supporting Information Fig. S6. The quantification of the bending angles is shown in Fig. S8. Bars, 100 μM.

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Fig. 4 Starch accumulation within the root apex and root gravitropic responses of several single mutant lines with disruptions in starch granule synthesis genes. **(**a-h) Lugol's staining (upper panel) and mPS-PI staining (lower panel) of the root tips of wild type (a), *ss1 (*b), *ss2* (c), *ss3* (d), *Gbss1* (e), *ss4* (f), *pgm* (g) and *adg1* (h). (i) Quantification of the root gravitropic responses of wild type, *ss1*, *ss2*, *ss3*, *Gbss1*, *ss4*, *pgm* and *adg1* are shown in Supporting Information Fig. S12. Degrees of bending (mean ± SD) were calculated for 10 independent primary roots from each genotype. Asterisks denote the statistical significance of comparisons with wild type: ***, $P < 0.001$. Bars, 20 μm.

Fig. 5 Starch accumulation within the root apex relies on the TIR1/AFB-mediated auxin signaling pathway. (a-b) Starch granule accumulation, observed with Lugol's staining (upper panel) and mPS-PI staining (lower panel), in the root apices of untreated (left panel) and NAA-treated (right panel) wild-type (a) and *tir1/afb2/afb3* mutant (b) seedlings. (c) qRT-PCR analysis of three key starch granule synthesis genes, *PGM*, *ADG1* and *SS4*, in wild-type and *tir1/afb2/afb3* mutant root tips with or without NAA treatment. (d-e) Lugol's staining (upper panel) and mPS-PI staining (lower panel) of untreated (left panel) and cvxIAA-treated root apices of the wild type (d) and the transgenic line *ccvTIR1* (e). (f) qRT-PCR analysis of *PGM*, *ADG1* and *SS4* in wild type and the transgenic line *ccvTIR1* with or without cvxIAA treatment. Error bars represent the ±SD from three biological replicates. Asterisks denote the statistical significance of comparisons with the untreated controls: ***, $P < 0.001$. Bars, 20 μm.

Fig. 6 The regulation of starch granule accumulation by auxin maxima is mediated by AXR3. (a) The expression of *DR5::GFP* in the gain-of-function mutant *axr3-1*. (b) The GFP channel image of (a) is shown in pseudocolor, and the intensity scale is shown at the right (H, high; L, low). (c) Defective starch granule accumulation in the *axr3-1* mutant. (d) Gravitropic response of WT and *axr3-1* mutant seedlings. (e) qRT-PCR analysis of three key starch granule

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synthesis genes, *PGM*, *ADG1* and *SS4*, in *axr3-1* mutant and wild-type root tips. Error bars represent ±SD from three biological replicates. Asterisks denote the statistical significance of comparisons with wild type: ***, $P < 0.001$. (f) Lugol's staining (upper panel) and mPS-PI staining (lower panel) of root tips of WT plants after incubation at 25°C or 37°C. (g) Lugol's staining (upper panel) and mPS-PI staining (lower panel) of root tips of *HS::axr3-1* after incubation at 25°C or 37°C. (h) qRT-PCR analysis of *PGM*, *ADG1* and *SS4* in WT root tips after incubation at 25°C or 37°C. (i) qRT-PCR analysis of *PGM*, *ADG1* and *SS4* in *HS::axr3-1* root tips after incubation at 25°C or 37°C. Error bars represent ±SD from three biological replicates. Asterisks denote the statistical significance of comparisons with wild type: ***, $P < 0.001$; **, $P < 0.01$. (j) GFP-enhancer trap line *Q1630* showing the S1/S2 layers of the columella cells in the wild-type and the *axr3-1* mutant root tips. (k) Comparison of the transcription levels of the auxin transporter genes *PIN1*, *PIN2*, *PIN3* and *PIN4* in wild type and the *axr3-1* mutant. Error bars represent ±SD from three biological replicates. (l-m) The expression patterns of PIN1-GFP (l) and PIN2-GFP (m) in wild-type and *axr3-1* mutant root tips. (n-o) *DR5::GFP* expression in the wild type (n) and the *axr3-1* mutant (o) with or without 1 μM NAA treatment for 48 h. p, Lugol's staining of the *axr3-1* mutant root tip with or without 1 μM NAA treatment for 48 h. WT, wild type. Bars, 20 μM.

Fig. 7 A working model of the dual role of auxin in mediating root gravitropism. The red lines indicate the role of auxin in mediating gravity perception by regulating starch granule accumulation, and the black lines indicate the role of auxin in mediating gravitropic response.

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Supporting Information

Fig. S1 Analysis of auxin maximum after application of auxin analogue and auxin inhibitor.

Fig. S2 The quantification of the amyloplast size shown in the lower panel of Fig. 1a-e (a) and h-j (b).

Fig. S3 Schematic diagram of the starch granule synthesis and degradation pathways.

Fig. S4 qRT-PCR analysis of the other starch granule synthesis (a) and degradation (b) genes in wild type root tips after NAA, NPA, L-Kyn and both L-Kyn/NAA treatment.

Fig. S5 Prediction of auxin responsive elements (ARE) at the promoter of the starch granule synthesis genes *PGM*, *ADG1* and *SS4.*

Fig. S6 The defective root gravitropism of auxin transporter *pin2* mutant and *pin3/4/7* triple mutant.

Fig. S7 Auxin maximum in *pin* mutants.

Fig. S8 The quantification of the amyloplast size shown in the lower panel of Fig.2b-f.

Fig. S9 The quantification of the root gravitropic angle in the Fig. 3f.

Fig. S10 Analysis of *DR5::GFP* expression with the root apex of 7-day-old wild-type *Arabidopsis* seedlings after the pre-treatment.

Fig. S11 The quantification of the amyloplast size shown in the lower panel of Fig.4a-h.

Fig. S12 The root gravitropic response of the 7-d-old wild type and starch granule synthase mutant seedlings.

Fig. S13 Lugol's staining of the WT, *pgm*, *adg1* and *ss4* mutant root tips with or without 1 μM NAA treatment.

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Fig. S14 The quantification of the amyloplast size shown in the lower panel of Fig.5a, b (a) and d, e (b).

Fig. S15 The increased starch granule accumulation (a) and accelerated root gravitropic response (b) of transgenic *Arabidopsis ccvTIR1* with pre-treatment of cvxIAA.

Fig. S16 Lugol's staining and mPS-PI staining of the WT and *pCEL5:ccvTIR1* after the 50 nM cvxIAA treatment.

Fig. S17 The quantification of the amyloplast size shown in the lower panel of Fig. S17a, b.

Fig. S18 The mPS-PI staining of the *axr3-1* mutant showed no starch granule accumulation in the columella cells.

Fig. S19 The quantification of the amyloplast size shown in the lower panel of Fig. 6f, g.

Fig. S20 The mPS-PI staining of columella cells of the *axr3-1* mutant with or without 1 μM NAA treatment.

Fig. S21 Lugol's staining and mPS-PI staining of the WT and transgenic line *pCEL5::axr3-1*.

Table. S1 List of primers used in this study.

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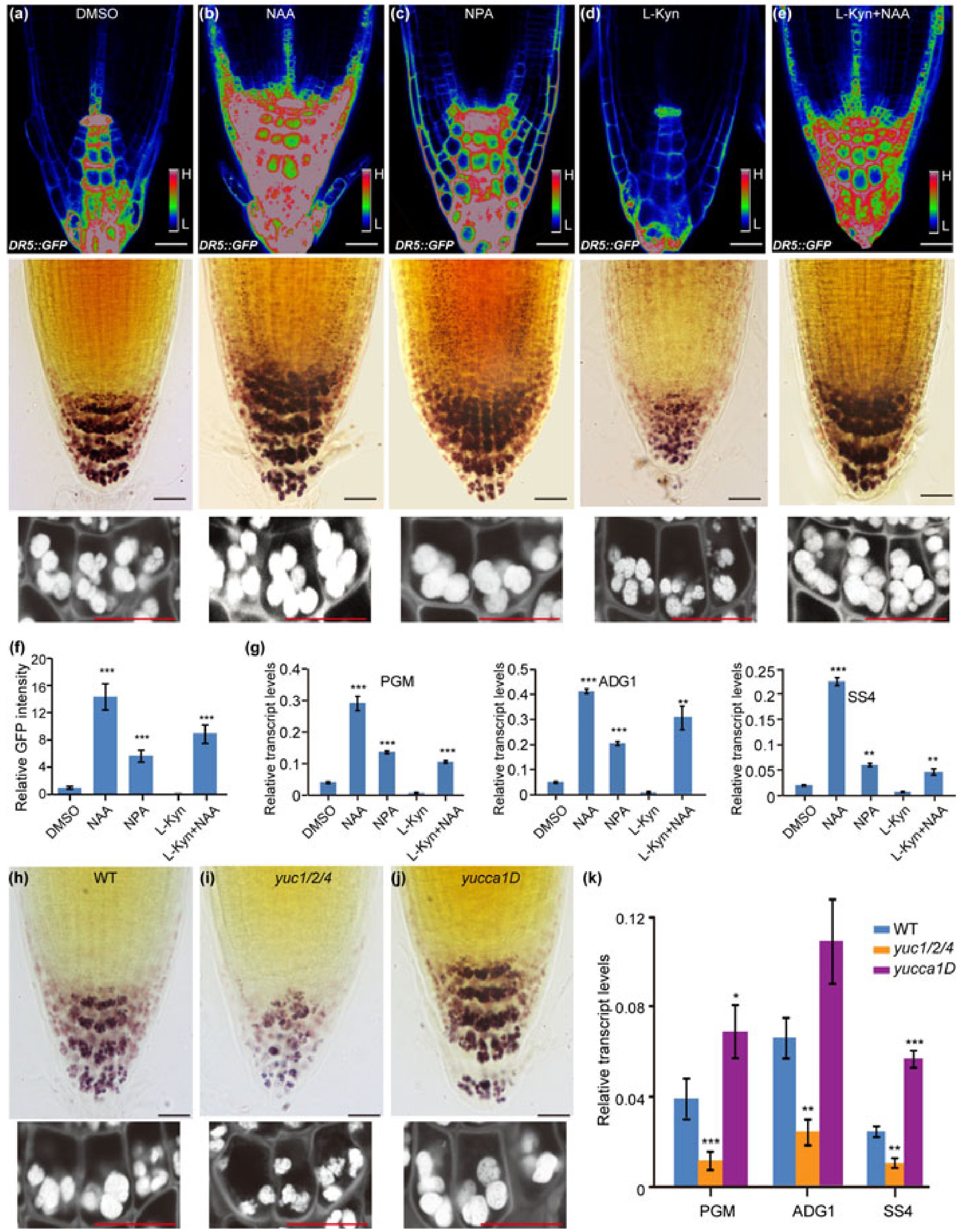

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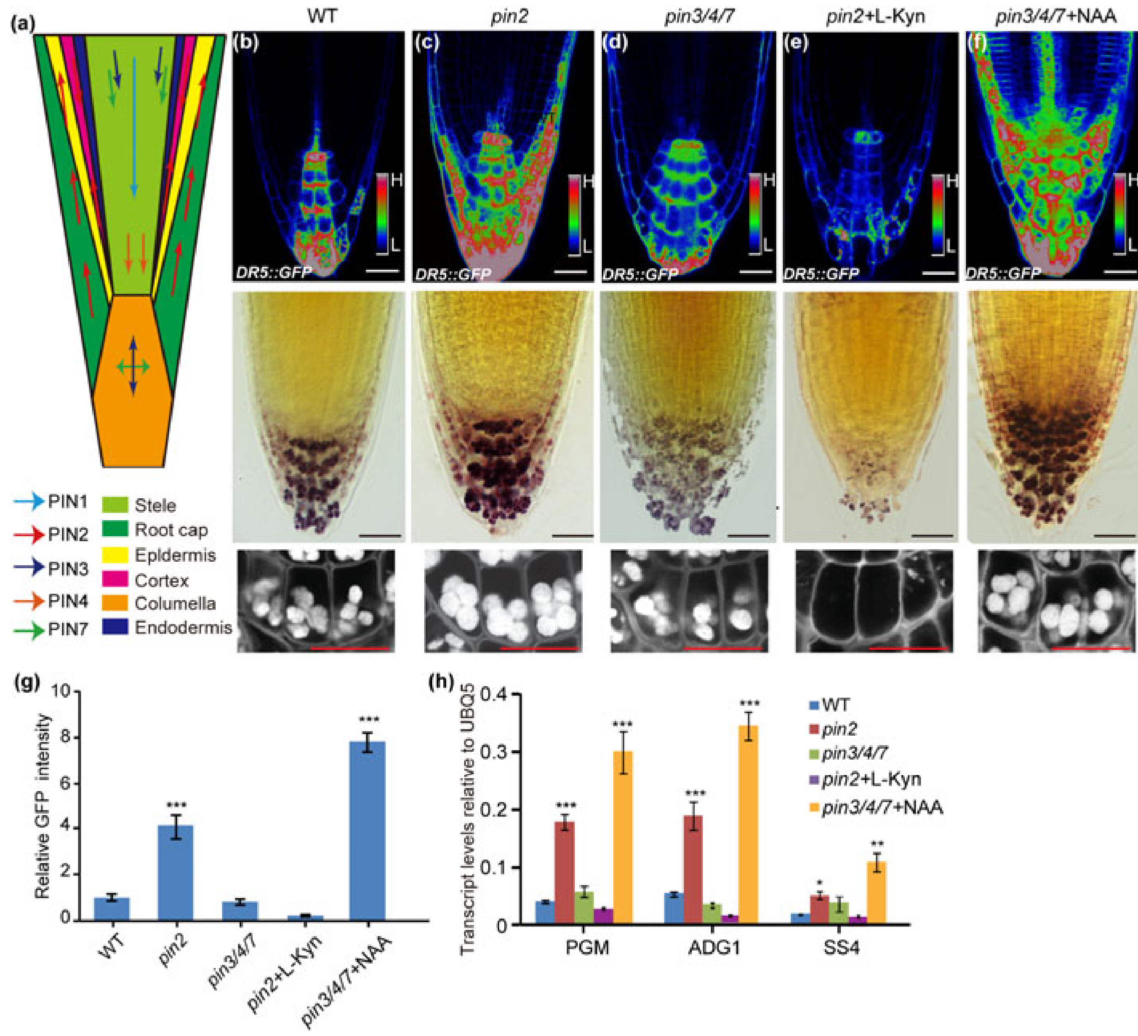

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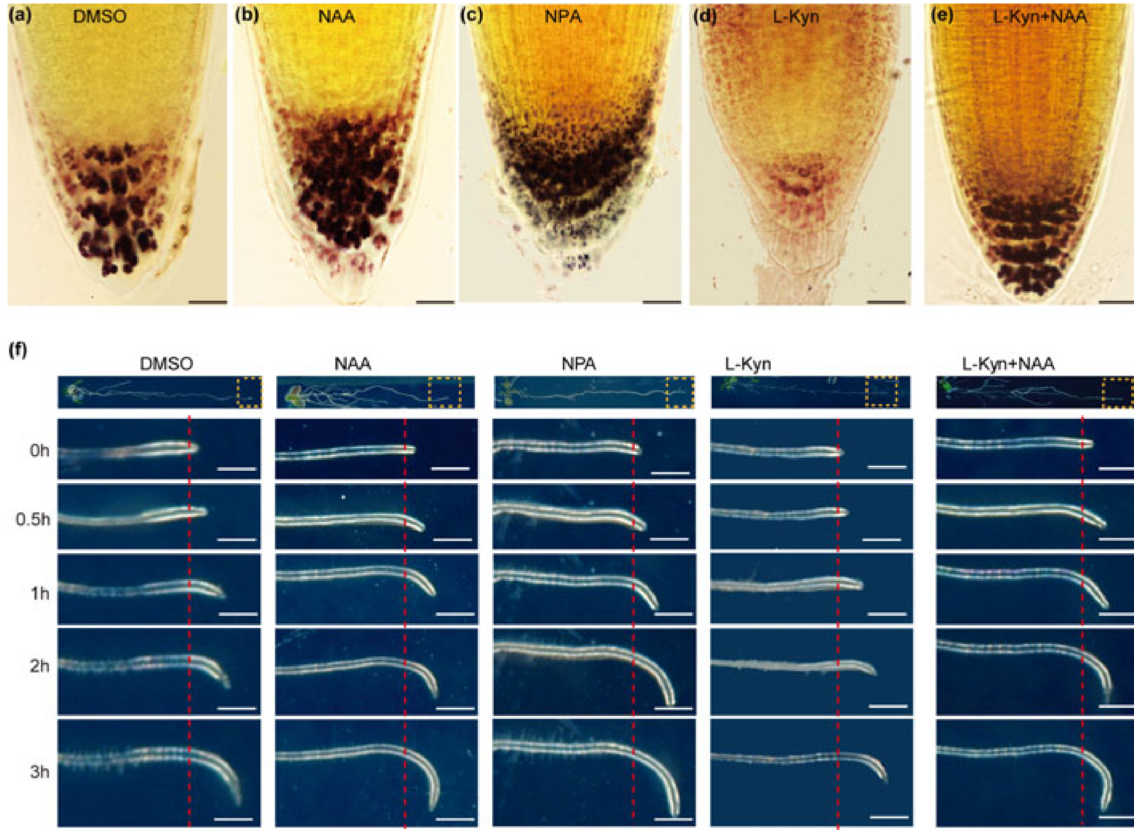

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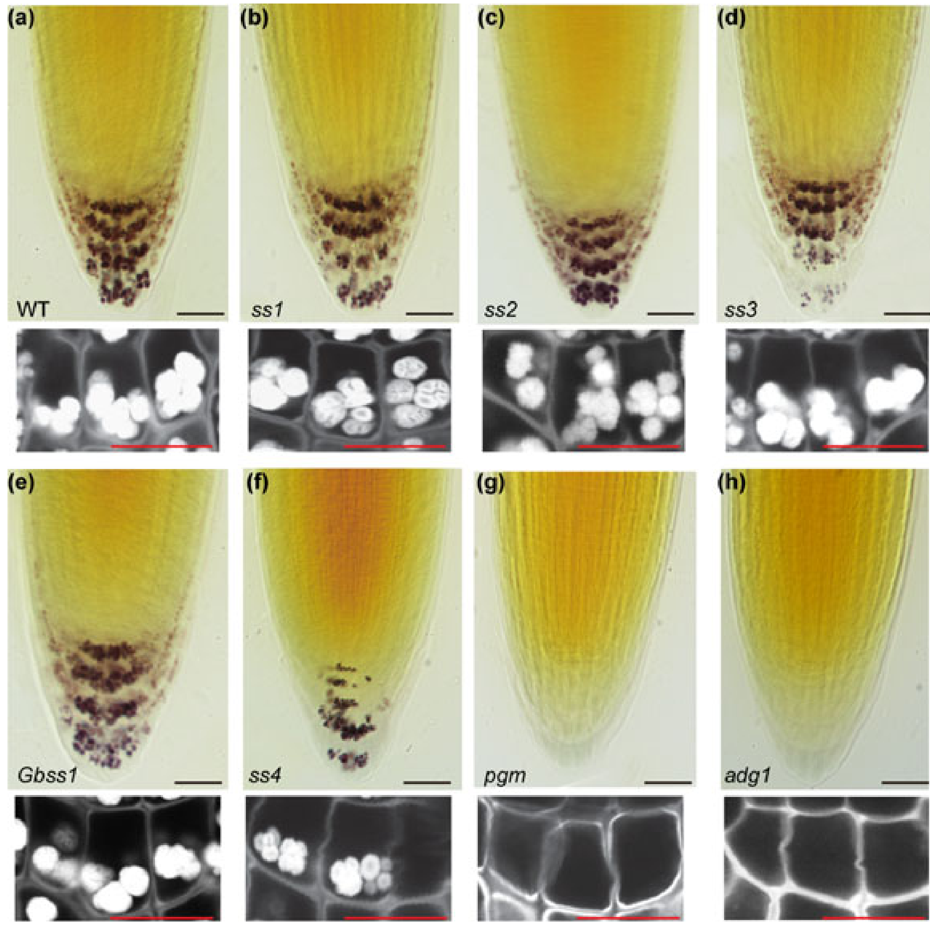

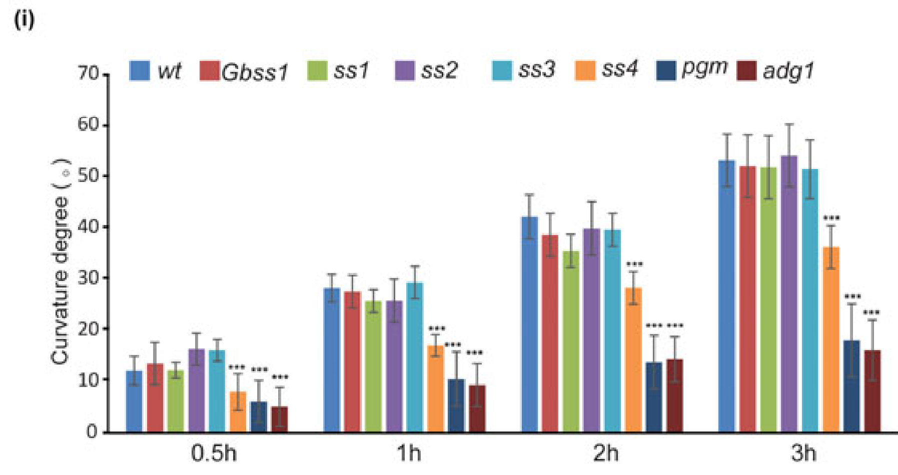

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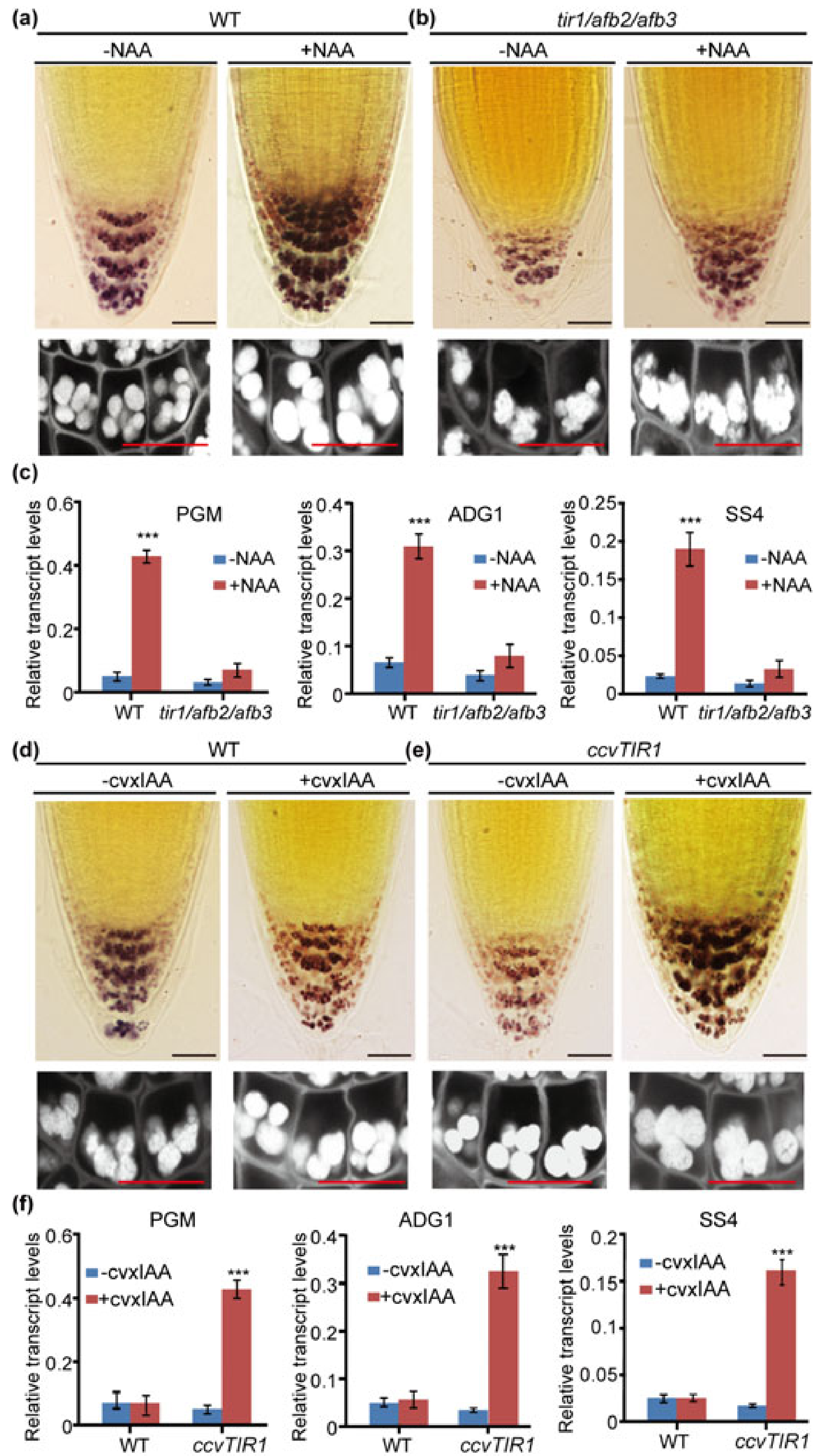

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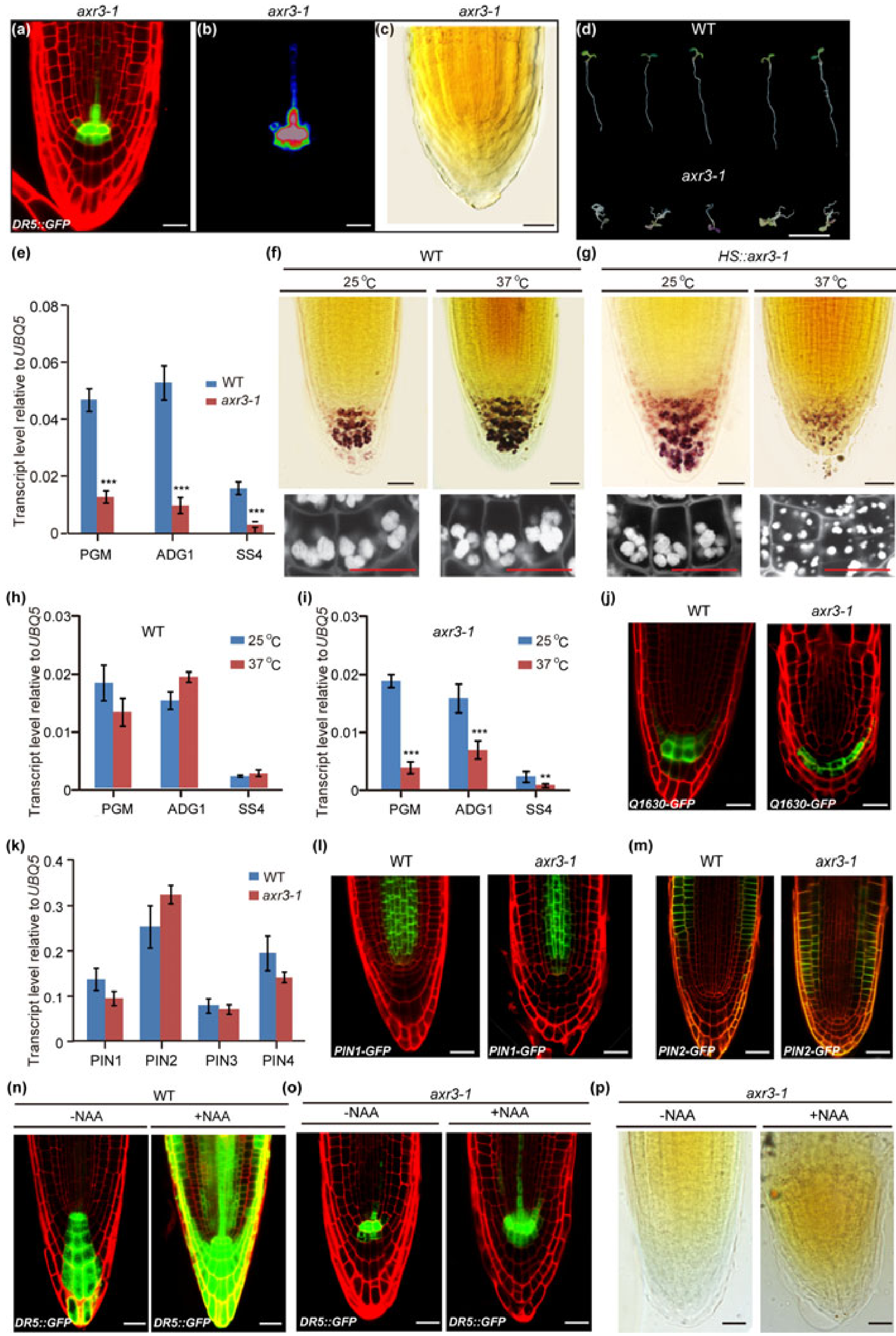

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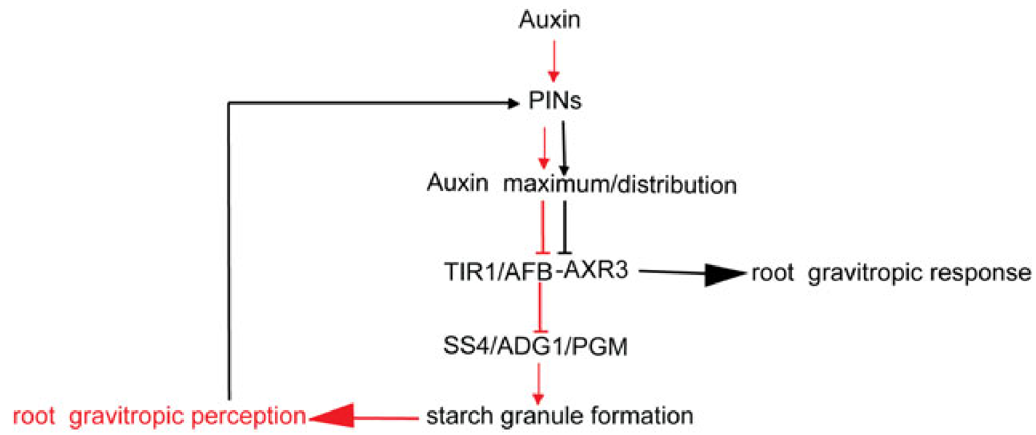

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